PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	)	
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Glenn J. Dorin, *et al.*	)	Examiner: TBA
	)	
Serial No. TBA	)	Group Art Unit: TBA
	)	
Filed: November 30, 2001	)	Atty. Dkt. No. 012441.00013

For: **Formulation, Solubilization, Purification, and Refolding of Tissue Factor Pathway Inhibitor (As Amended)**

PRELIMINARY AMENDMENT

The Honorable Assistant Commissioner for
Patents
Washington, D.C. 20231

Sir:

Applicants request entry of the following amendments prior to consideration of the application on the merits.

It is believed that a fee of $7968 for additional claims is required to file this amendment. Please charge our deposit account no. 19-0733 for the required amount.

IN THE TITLE

Page 1, line 1, please change the title as follows: Formulation, Solubilization, Purification, and Refolding of Tissue Factor Pathway Inhibitor

IN THE SPECIFICATION

Please insert the following paragraph as the first paragraph of page 1:

This application is a continuation of U.S. Serial No. 09/443,099, filed November 18, 1999, which is a divisional of U.S. Serial No. 09/973,211, filed June 11, 1999, pending, which is an application based on PCT/US96/09980, filed June 7, 1996, which is a continuation-in-part of U.S. Serial No. 08/473,668, filed June 7, 1995, now abandoned, which is a continuation-in-part of U.S. Serial No. 08/477,677, filed June 7, 1995, now abandoned.

Please amend the paragraph at page 6, line 16 as follows:

Figure 5 shows the solubility of TFPI at different pH conditions. About 10 mg/mL TFPI in 2M urea was dialyzed against 20 mM acetate, phosphate, citrate, glycine, L-glutamate or succinate in 150 mM NaCl. The concentration of remaining soluble TFPI after dialysis was measured by UV absorbance after filtering out the precipitates through 0.22 mm filter units.

Please amend the paragraph at page 6, line 27 as follows:

Figure 8 shows effect of pH on the stability of TFPI prepared in 10 mM Na phosphate, 150 mM NaCl and 0.005% (w/v) polysorbate-80. Stability samples containing 150 μg/mL TFPI were incubated at 40°C for 20 days. Kinetic rate constant for the remaining soluble TFPI was analyzed by following decrease of the main peak on cation exchange chromatograms.

Please amend the paragraph at page 7, line 1 as follows:

Figure 9 shows the percentage of remaining soluble TFPI measured by cation exchange HPLC (9A) and remaining active TFPI by prothrombin time assay (9B) as a function of phosphate concentration. The formulation contains 150 μg/mL TFPI prepared in 150 mM NaCl and 0.005% (w/v) polysorbate-80 at pH 7 with varying concentrations of phosphate.

Please amend the sequential paragraphs at page 7, line 24 as follows:

Figure 16 shows SDS PAGE analysis of fractions collected during elution of the S-Sepharose HP column used to purify rhTFPI from a polyphosphate-facilitated refold. Fig. 16A

shows Load through Fraction 23, and Fig. 16B shows Fractions 25-39.

Figure 17 shows the absorbance at 280 nm during the loading and elution of the Q-Sepharose HP column used to purify rhTFPI from a S-Sepharose pool prepared from a polyphosphate-facilitated refold.

Please amend the paragraph at page 8, line 1 as follows:

Figure 18 shows SDS PAGE analysis of fractions collected during elution of the Q-Sepharose HP column used to purify rhTFPI from a S-Sepharose pool prepared from a polyphosphate-facilitated refold. Fig. 18A shows Load through Fraction 19, and Fig. 18B shows Fractions 20-24.

Please amend the paragraph at Page 8, line 9 as follows:

Figure 21 shows SDS PAGE analysis of fractions collected during elution of the S-Sepharose HP column used to purify rhTFPI from a polyethyleneimine facilitated refold. Fig. 21A shows Load through Fraction 25, and Fig. 21B shows Fractions 26-Pool after fractions.

Please amend the paragraph at page 8, line 15 as follows:

Figure 23 shows SDS PAGE analysis of fractions collected during elution of the Q-Sepharose HP column used to purify rhTFPI from a S-Sepharose pool prepared from a polyethyleneimine-facilitated refold. Fig. 23A shows Load through Fraction 10, Fig. 23B shows Fractions 11-23, and Fig. 23C shows Fractions 24-30.

Please amend the paragraph at page 9, line 23 as follows:

The invention relates to pharmaceutically acceptable compositions wherein TFPI is present in a concentration of more than 0.2 mg/mL together with solubilizing agents. The solubilizing agents may be acetate ion, sodium chloride, citrate ion, isocitrate ion, glycine, glutamate, succinate ion, histidine, imidazole or sodium dodecyl sulfate (SDS) as well as charged polymers. In some compositions, TFPI may be present in concentrations of more than 1 mg/mL and more than 10 mg/mL. The composition may also have one or more secondary solubilizers. The secondary solubilizer or solubilizers may be polyethylene glycol (PEG), sucrose, mannitol,

or sorbitol. Finally, the composition may also contain sodium phosphate at a concentration greater than 20 mM.

Please amend the paragraph at page 14, line 1 as follows:

Charged polymers can be used to modify the charge, charge density, and reduce or eliminate ionically mediated limitations to conformation that may arise in the unfolded state. The juxtaposition of charged groups that are not normally in proximity may result in dead-end refolding pathways from which the refolding process may never recover.

Please amend the paragraph at page 18, line 24 as follows

The term "HIC" as used herein refers to hydrophobic interaction chromatography which employs a hydrophobic interaction between the column and the molecule of interest to separate the sulfated polysaccharides and other contaminants from the refolded product.

Please amend the paragraph at page 18, line 28 as follows:

Negatively charged polymers include sulfated polysaccharides, such as heparins, dextran sulfates, and agaropectins, as well as carboxylic acid polysaccharides such as alginic acids and carboxymethyl celluloses. Polyinorganics such as polyphosphates are also included. Polyamino acids such as polyasparatate, polyglutamate, and polyhistidine can also be used.

Please amend the paragraph at page 21, line 3 as follows:

TFPI can be prepared in yeast expression systems as described in U.S. Serial No. 08/286,530 (now abandoned, but continuation issued as U.S. 6,103,500), which is herein incorporated by reference. Methods have also been disclosed for purification of TFPI from yeast cell culture medium, such as in Petersen *et al*, J.Biol.Chem. 18:13344-13351 (1993). In these cases, recombinant TFPI is secreted from the yeast cell. TFPI recovered in such protocols is also frequently heterogeneous due to N-terminal modification, proteolytic degradation, and variable glycosylation. Therefore, a need exists in the art to produce mature TFPI that is authentic (i.e. having the correct N-terminal amino acid sequence), full-length and homogeneous.

Please amend the seqiential paragraphs at page 22, line 5 as follows:

TFPI activity may be tested by the prothrombin time assay (PTT assays). Bioactivity of TFPI was measured by the prothrombin clotting time using a model RA4 Coag-A-Mate from Organon Teknika Corporation (Oklahoma City, OK). TFPI samples were first diluted to 9 to 24 ug/mL with a TBSA buffer (50 mM Tris, 100 mM NaCl, 1 mg/mL BSA, pH 7.5) . Then 10 uL of Varify 1 (pooled normal plasma from Organon Teknika Corp.) was mixed with 90 uL of diluted TFPI samples in a sample tray and warmed to 37°C in the instrument. Finally Simplastin Excel (Thromboplastin from Organon Teknika Corp.) was added to start the clotting. The time delay in clotting due to anticoagulant activity of TFPI was measured and converted into TFPI concentration in the measured samples by comparison to a TFPI standard curve.

TFPI may be prepared by recombinant methods as disclosed in U.S. 5,212,091, the disclosure of which is herein incorporated by reference. Briefly, TFPI is expressed in *Escherichia coli* cells and the inclusion bodies containing TFPI are isolated from the rest of the cellular material. The inclusion bodies are subjected to sulfitolysis, purified using ion exchange chromatography, refolded by disulfide interchange reaction and the refolded, active TFPI purified by cation exchange chromatography. TFPI may also be puroduced in yeast as disclosed in U.S. 6,103,500.

Please amend the paragraph at page 25, line 1 as follows:

The refolding samples, stored at -20°C, remained in the standard refolding buffer containing 3 M urea, 50 mM Tris, pH 8.8, 1-4 mM redox, 0.5 mg/ml TFPI, and 0.2-0.6 M NaCl depending on condition. Samples refolded with dextran or heparin had 0.2 M salt, and samples without dextran or heparin had 0.6 M NaCl.

Please amend the paragraph at page 26, line 21 as follows:

About 10 mg/mL TFPI in 2M urea was dialyzed against one of the following: 20 mM acetate, 20 mM phosphate, 20 mM citrate, 20 mM glycine, 20 mM L-glutamate or 20 mM succinate in 150 mM NaCl as described above. 6-10 mg/mL TFPI bulk stock was loaded into Spec/Por 7 dialysis tubings (MW cutoff 3,500). Dialysis was carried out either at 4°C or ambient temperature. Three changes of buffer at a protein solution to buffer ratio: 1 to 50-100,

were made during course of dialysis over 12 to 24 hr time period. After dialysis, TFPI solution was filtered by Costar 0.22 micron filter units to separate precipitated TFPI from soluble TFPI. The solubility of TFPI was then measured by UV/Vis absorbance assuming an absorptivity 0.68 $(mg/mL)^{-1}$ cm^{-1} at 278 nm. The solutions were prepared at various pH levels by titration with HCl or NaOH.

Please amend the paragraph at page 27, line 1 as follows:

After completion of dialysis, the precipitates were filtered through 0.22 μm filter units. The concentration of remaining soluble TFPI after dialysis was measured by UV absorbance. Figure 5 shows the results of these experiments. Solubility of TFPI increased greatly in solutions containing 20 mM acetate, 20 mM phosphate, 20 mM L-glutamate and 20 mM succinate at pH levels below 7 and particularly at or below pH 4.5. Solubility of TFPI was also substantially increased in solutions containing 20 mM glycine above pH 10. Figure 6 shows the solubility of TFPI as a function of concentration of citrate ion in the presence of 10 mM Na phosphate at pH 7. TFPI solubility increases with increasing concentration of citrate. Figure 7 shows the solubility of TFPI as a function of concentration of NaCl at pH 7.0. TFPI solubility increases with increasing salt concentration, indicating salt promotes solubility of TFPI.

Please amend the lefthand column of Table 1 at page 30, lines 37 - 39, column 1 as follows:

20 mM Na Citrate, 130 mM NaCl, 1% Glycine, 0.25% Polysorbate-80,
5% PEG-400

20 mM Na Citrate, 130 mM NaCl, 1% Glycine, 0.25% Polysorbate-80

Please amend the paragraph at page 32, line 2 as follows:

The stability of TFPI stored at various pH conditions was tested. TFPI was prepared by dialysis as above in 10 mM Na phosphate, 150 mM NaCl and 0.005% (w/v) polysorbate-80. Stability samples containing 150 mg/mL TFPI were incubated at 40°C for 20 days. Kinetic rate constant for the remaining soluble TFPI was analyzed by following decrease of the main peak on cation exchange chromatograms. As can be seen in Figure 8, the decay rate constant increases at pH above 6.0, indicates more aggregation at higher pH conditions.

Please amend the sequential paragraphs at page 32, line 9 as follows:

TFPI was also formulated at a concentration of 150 mg/mL in 150 mM NaCl and 0.005% (w/v) polysorbate-80 at pH 7 with varying concentrations of phosphate. Figure 9A shows the percentage of remaining soluble TFPI measured by the cation exchange HPLC. Increasing concentrations of phosphate ion in solution resulted in higher levels of soluble TFPI remaining after incubation at 40°C. Higher levels of phosphate ion also resulted in higher levels of active TFPI as assayed by the prothrombin time assay. These results are shown in Figure 9B.

Stability of TFPI at a concentration of 0.5 mg/mL and formulated in 10 mM Na citrate, pH 6 and 150 mM NaCl was also tested at 40°C over a 20 day period. As seen in Figure 10, cation-exchange HPLC (triangle) shows the presence of soluble TFPI at levels greater than 60% initial, even after the 20 day incubation. In like manner, the prothrombin time assay (circle) shows the presence of active TFPI at levels greater than 60% initial, even after the 20 day incubation.

Please amend the paragraph at page 32, line 22 as follows:

Figure 11 shows loss of soluble TFPI at 40°C measured by both cation-exchange HPLC (open symbol) and prothrombin time assay (closed symbol) for 0.5 mg/mL TFPI formulated in 10 mM Na phosphate, pH 6 and either 150 mM NaCl (triangle) or 500 mM NaCl (circle).

Please amend the paragraph at page 32, line 26 as follows:

Figure 12 shows loss of soluble TFPI at 40°C measured by both cation-exchange HPLC (open symbol) and prothrombin time assay (closed symbol) for 0.5 mg/mL TFPI formulated in 10 mM Na acetate and pH 5.5 containing 150 mM NaCl (triangle) or 8% (w/v) sucrose (square) or 4.5% (w/v) mannitol (circle).

Please amend the paragraph at page 33, line 1 as follows:

Figure 13 shows two non-reducing SDS gels for TFPI formulation samples in 10 mM $NaPO_4$, 150 mM NaCl, and 0.005% polysorbate-80 at pH 4 to pH 9 stored at 40°C for 0 days (lower) and 20 days (upper). No loss on TFPI is seen at 0 days. However, at 20 days cleavage

fragments of TFPI may be seen at the lower pH range (i.e. pH 4 and pH 5). Without being bound to a particular theory, it is believed that these fragments may result from an acid catalyzed reaction.

Please amend the paragraph at page 37, line 5 as follows:

The acidified, filtered refold was loaded onto the equilibrated SP Sepharose HP column at a flow rate of approximately 80.0 ml/min. Flow rate was adjusted to accommodate overnight loading of the acidified filtered refold mixture. The column was equilibrated in 6 M urea, 20 mM sodium phosphate buffer pH 5.9 prior to loading. After loading, the column is washed with 2 CV of 6 M urea, 0.3 M NaCl, 20 mM sodium phosphate buffer, pH 5.9 prior to the gradient elution step. The column flow rate was increased to 190-200 ml/min for the wash step and all subsequent steps (linear velocity =~ 47 cm/hr). The product was eluted from the column using a linear salt gradient from 0.3 to 0.5 M NaCl in 6 M urea, 20 mM sodium phosphate buffer, pH 5.9. The gradient was formed by delivering 6 M urea, 0.5 M NaCl, 20 mM sodium phosphate buffer into 6 M urea, 0.3 M NaCl, 20 mM sodium phosphate buffer. Limit buffer was pumped with a Masterflex pump (model 7553-20) with a Masterflex head (model 7015.21) at a flow rate of approximately 100 ml/min. with vigorous mixing using a Paratrol A mixer from Parametrics (model 250210). The total volume of the gradient was 71.0 liters or 13.0 CV. The pH of the gradient buffers was 5.92 (+/- .02). Fractions are evaluated qualitatively using SDS PAGE and pooled based on the content of the correctly folded SC-59735 relative to other misfolds and impurities. After pooling the process stream is referred to as the S pool.

Please amend the paragraph at page 38, line 3 as follows:

An Amicon column (7.0 cm diameter) was packed with approximately 700 ml of Q-Sepharose high performance medium (Pharmacia Q-Sepharose HP). The column was packed with 20% ethanol at 20 psi. The bed height after packing was approximately 18 cm. The column was equilibrated with 5 CV of 6 M urea, 0.02 M Tris/HCl buffer, pH 8. The target for protein loading is 8-10 mg protein/ml Q Sepharose resin. The Q load was applied to the column at a flow rate 30-35 ml/min (50 cm/hr). After loading, the column was washed with approximately 5 CV of 6 M urea, 20 mM Tris/HCl buffer, pH 8.0, or until the absorbance at 280

nm returned to baseline. The product was eluted using a sodium chloride gradient from 0-0.15 M NaCl in 6 M urea, 20 mM Tris/HCl buffer, pH 8.0 over 25 column volumes. The first seven column volumes were collected as a single fraction, followed by 30 fractions of 0.25 column volume each.

Please amend the paragraph at page 39, line 24 as follows:

After approximately 96 h, the refolding process was terminated by adjusting the pH of the refold to pH 5.9 using glacial acetic acid. Stirring was continued for 90 minutes and the pH checked. More acid was added, if necessary to adjust the pH to 5.9 +/- 0.1.

Please amend the paragraph at page 41, line 6 as follows:

The pH of the S-pool was next adjusted to pH 8.0 with 2.5 N NaOH. The S Pool was concentrated 2-3 fold to approximately 2 L using an Amicon DC-10L ultrafiltration unit containing an Amicon YM10 spiral cartridge (10,000 N.W. cut-off membrane). After concentration, the concentrated S Pool was diafiltered against 7 volumes of 6 M urea, 20 mM Tris-HCl buffer, pH 8.0. The diafiltration was considered complete when the conductivity of the retentate was below 2 mS. The diafiltered concentrate was drained from the ultrafiltration unit and the unit was washed with approximately 1 L of diafiltration buffer. The wash is combined with the concentrate to form the Q-load.

Please amend the paragraph at page 41, line 15 as follows:

An Amicon column (7.0 cm diameter) was packed with approximately 700 ml of Q-Sepharose high performance medium (Pharmacia Q-Sepharose HP). The column was packed in 20% ethanol at 20 psi. The bed height after packing was approximately 18 cm. The column was equilibrated with 5 CV of 6 M urea, 0.02 M Tris/HCl buffer, pH 8. The target for protein loading is 8-10 mg protein/ml Q Sepharose resin. The Q load was applied to the column at a flow rate 30-35 ml/min (50 cm/hr). After loading, the column was washed with approximately 5 CV of 6 M urea, 20 mM Tris/HCl buffer, pH 8.0, or until the absorbance at 280 nm returned to baseline. The product was eluted using a sodium chloride gradient from 0-0.15 M NaCl in 6 M urea, 20 mM Tris/HCl buffer, pH 8.0 over 25 column volumes. The first seven column volumes

were collected as a single fraction, followed by 30 fractions of 0.25 column volume each.

Please amend the paragraph at page 42, line 1 as follows:

The Q-Sepharose fractions to be pooled were thawed by incubation at 2-8°C, pooled, and the pH of the pool was adjusted to 7.2 using 2 M HCl. The pool was then concentrated approximately 5 fold in an Amicon DC-1 ultrafiltration system containing a S1Y1 Amicon YM-10 cartridge (10,000 MWCO spiral cartridge membrane). The concentrated Q Pool was then diafiltered against seven column volumes of 2 M urea, 0.15 M NaCl, 20 mM sodium phosphate buffer, pH 7.2. Following ultrafiltration, the solution was drained from the ultrafiltration system. Approximately 100 ml of 2 M urea, 0.15 M NaCl, 20 mM sodium phosphate buffer, pH 7.2 was circulated through the ultrafiltration system for approximately 5 min. The rinse solution was combined with the original concentrate and filtered through a 0.45 micron vacuum filter unit (Nalgene).

Please amend the paragraph at page 42, line 16 as follows:

About 2 g of rhTFPI (43 ml inclusion body slurry containing 46 mg/ml rhTFPI) was dissolved with mixing in 4 L of 50 mM Tris buffer, pH 10.5 containing 4 g/l polyphosphate (Glass H, FMC Corporation) at 2-8°C. Sufficient cysteine and cystine was added to make the solutions 0.1 mM and 0.05 mM respectively. The pH was maintained at pH 10.5 with 1 N NaOH. The refold solution was incubated at 2-8°C with gentle mixing for 72-96 h.

IN THE CLAIMS

Please cancel claims 1-70 without prejudice.

Please add the following new claims 71-262.

71. A solution having a pH of from 5 to 10, comprising from 200 mM arginine to 300 mM arginine, and further comprising a polypeptide selected from the group consisting of (i) human TFPI having the amino acid sequence shown in Figure 4, (ii) ala-human TFPI having the

amino acid sequence shown in Figure 4 and having one further amino acid which is an amino-terminal alanine, and (iii) muteins of (i) or (ii) having from 1 to 5 amino acid substitutions.

72. The solution of claim 71 wherein said arginine is L-arginine.

73. The solution of claim 71 comprising from 0.2 to 10 mg/ml of said polypeptide.

74. The solution of claim 72 wherein the polypeptide is ala-human TFPI having the amino acid sequence shown in Figure 4 and having one further amino acid which is an amino-terminal alanine.

75. The solution of claim 71 comprising 300 mM arginine.

76. The solution of claim 74 having a citrate/citric acid buffer at a total buffer concentration of from 5 mM to 300 mM.

77. The solution of claim 71 having a pH of 5.5 and comprising ala-human TFPI having the amino acid sequence shown in Figure 4 and having one further amino acid which is an amino-terminal alanine, 300 mM L-arginine, and a citrate/citric acid buffer at a total buffer concentration of 20 mM.

78. A solution having a pH of from 5 to 10, comprising more than 0.2 mg/ml of a polypeptide selected from the group consisting of (i) human TFPI having the amino acid sequence shown in Figure 4, (ii) ala-human TFPI having the amino acid sequence shown in Figure 4 and having one further amino acid which is an amino-terminal alanine, and (iii) muteins of (i) or (ii) having from 1 to 5 amino acid substitutions, and further comprising a solubilizer selected from the group consisting of sucrose, mannitol, sorbitol, citrate, isocitrate, succinate, malate, polyphosphate, sodium phosphate, sodium sulfate, acetate, polysorbate-80, polyethylene glycol, histidine, imidazole, glutamate, glycine, ammonium sulfate, and sodium dodecyl sulfate.

79. The solution of claim 78 wherein the polypeptide is present at a concentration of from 1 to 20 mg/ml.

80. The solution of claim 79 wherein the polypeptide is present at a concentration of from 1 to 10 mg/ml.

81. The solution of claim 78 further comprising sodium phosphate at a concentration greater than 20mM.

82. The solution of claim 78 wherein the solution comprises 0.5M sodium phosphate.

83. The solution of claim 78 wherein the solution comprises 0.5M sodium citrate.

84. The solution of claim 78 wherein the solution comprises 0.5M sodium isocitrate.

85. The solution of claim 78 wherein the pH of the solution is below pH 7.0, and wherein the solubilizer is not glycine.

86. The solution of claim 78 wherein the solubilizer is acetate ion and the acetate ion is present in the solution at a concentration of from 5 mM to 200 mM.

87. The solution of claim 78 wherein the solubilizer is citrate ion and the citrate ion is present in the solution as sodium citrate or potassium citrate at a concentration from 50 mM to 500 mM.

88. The solution of claim 78 wherein the solubilizer is isocitrate ion and the isocitrate ion is present in the solution as sodium isocitrate or potassium isocitrate at a concentration from 100 mM to 500 mM.

89. The solution of claim 78 wherein the solubilizer is glycine and the glycine is present in the solution at a concentration from 5 mM to 20 mM.

90. The solution of claim 78 wherein the solubilizer is glutamate and the glutamate is present in the solution at a concentration from 5 mM to 20 mM.

91. The solution of claim 78 wherein the solubilizer is succinate ion and the succinate ion is present in the solution as sodium succinate at a concentration from 5 mM to 20 mM.

92. The solution of claim 78 wherein the solubilizer is histidine and the histidine is present in the solution at a concentration from 5 mM to 20 mM.

93. The solution of claim 78 wherein the solubilizer is imidazole and the imidazole is present in the solution at a concentration from 5 mM to 20 mM.

94. The solution of claim 78 wherein the solubilizer is sodium dodecyl sulfate and the sodium dodecyl sulfate is present in the solution at a concentration of 0.001 % to 0.1 % (weight / volume).

95. The solution of claim 78 wherein the solubilizer is polyethylene glycol and the polyethylene glycol is present in the solution at a concentration of 0.2 % to 10 % (weight / volume).

96. The solution of claim 78 wherein the solubilizer is sucrose and the sucrose is present in the solution at a concentration of 0.2 % to 10 % (weight / volume).

97. The solution of claim 78 wherein the solubilizer is mannitol and the mannitol is present in the solution at a concentration of 1.0 % to 5.0 % (weight / volume).

98. The solution of claim 78 wherein the solubilizer is sorbitol and the sorbitol is present in the solution at a concentration of 0.2 % to 10 % (weight / volume).

99. The solution of claim 78 comprising at least 20 mM citrate.

100. The solution of claim 78 comprising at least 5 mM sodium acetate.

101. The solution of claim 78 comprising at least 0.005% (w/v) polysorbate-80.

102. The solution of claim 78 comprising at least 5 mM histidine.

103. The solution of claim 78 comprising at least 10 mM imidazole.

104. The solution of claim 78 comprising at least 1% (w/v) glutamate.

105. The solution of claim 78 comprising at least 0.1% (w/v) polyphosphate.

106. The solution of claim 78 comprising at least 120 mM ammonium sulfate.

107. The solution of claim 78 comprising at least 0.02% (w/v) sodium dodecyl sulfate.

108. A solution according to any one of claims 78-107 wherein the polypeptide is ala-human TFPI having the amino acid sequence shown in Figure 4 and having one further amino acid which is an amino-terminal alanine.

109. A solution according to claim 108 which is pharmaceutically acceptable.

110. A solution according to any one of claims 78-107 which is pharmaceutically acceptable.

111. An aqueous composition comprising (1) a polypeptide selected from the group consisting of (i) human TFPI having the amino acid sequence shown in Figure 4, (ii) ala-human TFPI having the amino acid sequence shown in Figure 4 and having one further amino acid which is an amino-terminal alanine, and (iii) muteins of (i) or (ii) having from 1 to 5 amino acid substitutions, and (2) a stabilizer of the polypeptide, wherein the half-life at 40°C of the polypeptide in said composition, as determined using prothrombin time, is at least 20 days.

112. The composition of claim 111 wherein the half life is from 20 to about 70 days.

113. The composition of claim 112 wherein the pH of the composition is from 5 to 10.

114. The composition of claim 113 wherein the stabilizer is selected from the group consisting of sodium chloride at a concentration of at least 150 mM, sucrose at a concentration of at least 8% (weight/volume), and mannitol at a concentration of at least 4.5% (weight/volume).

115. The composition of claim 114 wherein the stabilizer is sodium chloride at a concentration of at least about 150 mM.

116. The composition of claim 115 further comprising 10 mM sodium citrate.

117. The composition of claim 116 having a pH of about 5.5.

118. The composition of claim 115 having a sodium chloride concentration of at least about 500 mM sodium chloride.

119. The composition of claim 118 further comprising a sodium phosphate buffer at a total buffer concentration of about 10 mM.

120. The composition of claim 119 having a pH of about 6.0.

121. The composition of claim 114 wherein the stabilizer is sucrose at a concentration of at least 8% (weight/volume).

122. The composition of claim 121 further comprising 10 mM sodium acetate.

123. The composition of claim 122 having a pH of about 5.5.

124. The composition of claim 114 wherein the stabilizer is mannitol at a concentration of at least 4.5% (weight/volume).

125. The composition of claim 124 further comprising 10 mM sodium acetate.

126. The composition of claim 125 having a pH of about 5.5.

127. The composition of claim 111 comprising 150 mM sodium chloride, 0.005% (weight/volume) polysorbate-80, and sodium phosphate buffer at a concentration of at least 50 mM and having a pH of 7.

128. A composition of any of claims 111-127 wherein the polypeptide is ala-human TFPI having the amino acid sequence shown in Figure 4 and having one further amino acid which is an amino-terminal alanine.

129. A method of increasing the solubility of a polypeptide selected from the group consisting of (i) human TFPI having the amino acid sequence shown in Figure 4, (ii) ala-human TFPI having the amino acid sequence shown in Figure 4 and having one further amino acid which is an amino-terminal alanine, and (iii) muteins of (i) or (ii) having from 1 to 5 amino acid substitutions, comprising the step of:

preparing an aqueous composition comprising the polypeptide and a charged polymer.

130. The method of claim 129 wherein the charged polymer is selected from the group consisting of dextran sulfate, glycosaminoglycan, heparin, polyaspartate, polyglutamate, agaropectin, alginic acid, carboxymethyl cellulose, polyphosphate, polyethyleneimine, polyethyleneimine cellulose, DEAE dextran, polylysine and polyarginine.

131. The method of claim 130 wherein the charged polymer is polyphosphate.

132. The method of claim 129 wherein the aqueous composition comprises at least about 0.5 mg/ml of the polypeptide.

133. The method of claim 131 wherein the polyphosphate is added to a final concentration of from 1 to 20 mg/ml.

134. The method of claim 131 wherein the aqueous composition comprises 3 M urea and 50 mM Tris at a pH of 10.5.

135. The method of claim 131 wherein the aqueous composition comprises at least about 0.5 mg/ml polypeptide, 4 mg/ml polyphosphate, 0.1 mM cysteine, 0.05 mM cystine, and 50 mM Tris at a pH of about 10.5.

136. The method of claim 131 wherein polyphosphate is added to a final concentration providing a weight ratio of the polypeptide to polyphosphate of from about 2 to 1 to about 1 to 8.

137. The method of claim 136 wherein, prior to adding polyphosphate, the polypeptide is present in a solution comprising 6 M urea, 125 mM sodium chloride, and 20 mM sodium phosphate buffer, said solution having a pH of about 7.4.

138. The method of claim 136 further comprising the step of:

removing low molecular weight solutes from the composition comprising the polypeptide and polyphosphate, to form an aqueous composition comprising the polypeptide and

polyphosphate essentially free of other solutes, wherein the concentration of the polypeptide in the composition is greater than 0.5 mg/ml.

139. The method of claim 129 wherein the polypeptide is present in an insoluble form prior to preparing the aqueous composition.

140. The method of claim 139 wherein the insoluble form is an inclusion body.

141. The method of claim 129 wherein the aqueous composition further comprises a chaotrope.

142. The method of claim 129 further comprising the step, prior to preparing the aqueous composition, of:

applying the polypeptide to a solid support.

143. The method of claim 142 wherein the aqueous composition is formed by adding polyphosphate in a concentration gradient to effect selective elution of the polypeptide from the solid support.

144. The method of claim 142 wherein the solid support is an ion exchange resin.

145. The method of claim 144 wherein the resin has a negative net charge.

146. The method of claim 144 wherein the resin has a positive net charge.

147. The method of claim 129 further comprising the step, after the step of preparing the aqueous composition, of:

applying the polypeptide to a solid support.

148. The method of claim 147 wherein the solid support is an ion exchange resin.

149. The method of claim 148 wherein the resin has a net negative charge.

150. The method of claim 148 wherein the resin has a positive net charge.

151. A method of any of claims 129-150 wherein the polypeptide is ala-human TFPI having the amino acid sequence shown in Figure 4 and having one further amino acid which is an amino-terminal alanine.

152. A method to aid in refolding a polypeptide selected from the group consisting of (i) human TFPI having the amino acid sequence shown in Figure 4, (ii) ala-human TFPI having the amino acid sequence shown in Figure 4 and having one further amino acid which is an amino-terminal alanine, and (iii) muteins of (i) or (ii) having from 1 to 5 amino acid substitutions, comprising the step of:

preparing an aqueous composition comprising the polypeptide and a charged polymer.

153. The method of claim 152 wherein the charged polymer is selected from the group consisting of dextran sulfate, glycosaminoglycan, heparin, polyaspartate, polyglutamate, agaropectin, alginic acid, carboxymethyl cellulose, polyphosphate, polyethyleneimine, polyethyleneimine cellulose, DEAE dextran, polylysine and polyarginine.

154. The method of claim 153 wherein the charged polymer is polyphosphate.

155. The method of claim 152 wherein the solution further comprises urea.

156. The method of claim 152 wherein the solution is essentially free of urea.

157. The method of claim 152 wherein the solution has a pH of from about 9 to at least about 11.

158. The method of claim 152 wherein the polypeptide is allowed to refold for at least about 72 hours.

159. The method of claim 152 wherein the refolding process is terminated after from about 72 hours to about 120 hours by lowering the pH of the solution to 5.9 +/- 0.1.

160. The method of claim 155 wherein refolding occurs in a solution comprising about 1 mg/ml polypeptide, about 2 mg/ml polyphosphate, 3 M urea, 0.1 mM cysteine, and 50 mM Tris, having a pH of about 10.5.

161. The method of claim 156 wherein refolding occurs in a solution comprising about 0.5 mg/ml polypeptide, about 4 mg/ml polyphosphate, 0.1 mM cysteine, 0.05 mM cystine, and 50 mM Tris, having a pH of about 10.5.

162. A method of obtaining a purified polypeptide selected from the group consisting of (i) human TFPI having the amino acid sequence shown in Figure 4, (ii) ala-human TFPI having the amino acid sequence shown in Figure 4 and having one further amino acid which is an amino-terminal alanine, and (iii) muteins of (i) or (ii) having from 1 to 5 amino acid substitutions, comprising the steps of:

refolding the polypeptide in the presence of a charged polymer; and

purifying the polypeptide using a method selected from the group consisting of cation exchange chromatography, anion exchange chromatography, and hydrophobic interaction chromatography.

163. A method to aid in solubilizing a polypeptide selected from the group consisting of (i) human TFPI having the amino acid sequence shown in Figure 4, (ii) ala-human TFPI having the amino acid sequence shown in Figure 4 and having one further amino acid which is an amino-terminal alanine, and (iii) muteins of (i) or (ii) having from 1 to 5 amino acid substitutions, comprising the step of:

suspending an insoluble form of said polypeptide in a solubilization solution comprising dithiothreitol at a concentration of from 5 to 50 mM.

164. The method of claim 163 wherein the insoluble form is an inclusion body.

165. The method of claim 163 wherein the solubilization solution comprises 10 mM dithiothreitol.

166. The method of claim 165 wherein the solubilization solution comprises 8 M urea and 50 mM Tris and has a pH of 8.5.

167. The method of claim 163 wherein the solubilization solution comprises 50 mM dithiothreitol.

168. The method of claim 167 wherein the solubilization solution comprises 3.5 M guanidinium hydrochloride and 50 mM Tris and has a pH of 7.1.

169. A method of any of claims 163-168 wherein the polypeptide is ala-human TFPI having the amino acid sequence shown in Figure 4 and having one further amino acid which is an amino-terminal alanine.

170. A method of removing bacterial contaminants from a recombinant polypeptide expressed in bacteria, wherein said recombinant polypeptide is selected from the group consisting of (i) human TFPI having the amino acid sequence shown in Figure 4, (ii) ala-human TFPI having the amino acid sequence shown in Figure 4 and having one further amino acid which is an amino-terminal alanine, and (iii) muteins of (i) or (ii) having from 1 to 5 amino acid substitutions, comprising the steps of:

adding a cationic polymer to a solution comprising a soluble form of said recombinant polypeptide, to precipitate bacterial contaminants; and

removing the precipitated bacterial contaminants.

171. The method of claim 170 wherein the step of adding comprises diluting a solution comprising a soluble form of polypeptide at least ten-fold into a solution comprising 0.5% (weight/volume) of the cationic polymer.

172. The method of claim 170 wherein the cationic polymer is Betz polymer 624.

173. The method of claim 170 wherein, prior to addition of the charged polymer, the solution comprises 3.5 M guanidinium hydrochloride and 50 mM dithiothreitol, and 50 mM Tris and has a pH of 7.1.

174. The method of claim 170 wherein the precipitate is removed by centrifugation.

175. The method of claim 170 wherein the precipitate is removed by filtration.

176. The method of claim 170 wherein the recombinant polypeptide was expressed in Escherichia coli.

177. The method of claim 170 wherein the recombinant polypeptide is incorporated into inclusion bodies when expressed.

178. A method of any of claims 170-177 wherein the polypeptide is ala-human TFPI having the amino acid sequence shown in Figure 4 and having one further amino acid which is an amino-terminal alanine.

179. A method of purifying a polypeptide expressed in Escherichia coli, wherein said polypeptide is selected from the group consisting of (i) full length, properly folded human TFPI having the amino acid sequence shown in Figure 4; (ii) full length, properly folded ala-human TFPI having the amino acid sequence shown in Figure 4 and having one further amino acid which is an amino-terminal alanine; and (iii) a full length, properly folded mutein selected from

the group consisting of muteins having from 1 to 5 amino acid substitutions of (i) or (ii); the method comprising the steps of:

applying a first preparation comprising said polypeptide to a hydrophobic interaction chromatography resin to bind said polypeptide to the resin; and

washing the resin with an elution buffer having a lower salt concentration than said first preparation to elute from the resin a second preparation enriched in said polypeptide compared to said first preparation.

180. The method of claim 179 wherein the polypeptide is full length, properly folded human TFPI having the amino acid sequence shown in Figure 4

181. The method of claim 179 wherein the polypeptide is full length, properly folded ala-human TFPI having the amino acid sequence shown in Figure 4 and having one further amino acid which is an amino-terminal alanine.

182. The method of claim 179 wherein the first preparation comprises urea.

183. The method of claim 182 wherein the first preparation comprises about 3 M urea.

184. The method of claim 179 wherein the first preparation comprises about 1.2 M ammonium sulfate.

185. The method of claim 179 wherein the first preparation comprises about 0.6 M ammonium sulfate.

186. The method of claim 179 wherein the first preparation has a pH of from about pH 5.5 to about pH 6.0.

187. The method of claim 186 wherein the first preparation has a pH of about 5.7.

188. The method of claim 179 wherein the first preparation comprises about 3 M urea, about 1.2 M ammonium sulfate, about 80 mM NaCl, about 20 mM Tris, and has a pH of about 5.68.

189. The method of claim 179 wherein the elution buffer comprises a gradient of decreasing salt concentration.

190. The method of claim 179 wherein the elution buffer comprises a gradient of decreasing ammonium sulfate concentration.

191. The method of claim 190 wherein the gradient starts at about 1 M ammonium sulfate and ends at about 0 M ammonium sulfate.

192. The method of claim 190 wherein the gradient starts at about 0.5 M ammonium sulfate and ends at about 0 M ammonium sulfate.

193. The method of claim 190 wherein the gradient volume is about 5 column volumes.

194. The method of claim 179 wherein the elution buffer comprises urea.

195. The method of claim 179 wherein the elution buffer comprises about 3 M urea.

196. The method of claim 179 wherein the elution buffer comprises about 3 M urea, a gradient from about 1 M ammonium sulfate to about 0 M ammonium sulfate, about 0.1 M of a buffer, and has a pH of about 6.0.

197. The method of claim 179 wherein the elution buffer comprises about 3 M urea, a gradient from about 0.5 M ammonium sulfate to about 0 M ammonium sulfate, about 0.1 M of a buffer, and has a pH of about 6.0.

198. The method of claim 196 or 197, wherein the buffer comprises about 33 mM of MES, about 33 mM of HEPES, and about 33 mM of sodium acetate.

199. The method of claim 179 wherein the hydrophobic interaction chromatography resin is a butyl resin.

200. The method of claim 199 wherein the resin beads have a diameter of about 4.6 microns and a length of about 100 microns.

201. A method of purifying a polypeptide expressed in Escherichia coli, wherein said polypeptide is selected from the group consisting of (i) full length, properly folded human TFPI having the amino acid sequence shown in Figure 4; (ii) full length, properly folded ala-human TFPI having the amino acid sequence shown in Figure 4 and having one further amino acid which is an amino-terminal alanine; and (iii) a full length, properly folded mutein selected from the group consisting of muteins having from 1 to 5 amino acid substitutions of (i) or (ii); the method comprising the steps of:

applying a first preparation comprising said polypeptide to an anion exchange chromatography resin to bind said polypeptide to the resin;

washing the resin with a wash buffer; and

washing the resin with an elution buffer comprising a polyionic compound to elute from the resin a second preparation enriched in said polypeptide compared to said first preparation.

202. The method of claim 201 wherein the polypeptide is full length, properly folded human TFPI having the amino acid sequence shown in Figure 4

203. The method of claim 201 wherein the polypeptide is full length, properly folded ala-human TFPI having the amino acid sequence shown in Figure 4 and having one further amino acid which is an amino-terminal alanine.

204. The method of claim 201 wherein the first preparation comprises urea.

205. The method of claim 204 wherein the first preparation comprises about 6 M urea.

206. The method of claim 201 wherein the first preparation has a pH of about 8.

207. The method of claim 201 wherein the first preparation comprises about 6 M urea, about 20 mM Tris, and has a pH of about 8.

208. The method of claim 201 wherein the wash buffer comprises urea.

209. The method of claim 201 wherein the wash buffer comprises about 6 M urea.

210. The method of claim 201 wherein the wash buffer has a pH of about 9.

211. The method of claim 201 wherein the wash buffer comprises about 6 M urea, about 20 mM Tris, and has a pH of about 9.

212. The method of claim 201 wherein the elution buffer comprises urea.

213. The method of claim 201 wherein the elution buffer comprises about 6 M urea.

214. The method of claim 201 wherein the polyionic compound is a polyanion.

215. The method of claim 214 wherein the polyionic compound is polyphosphate.

216. The method of claim 215 wherein the elution buffer comprises about 10 mg/ml of polyphosphate.

217. The method of claim 201 wherein the elution buffer has a pH of about 9.

218. The method of claim 201 wherein the elution buffer comprises about 6 M urea, about 10 mg/ml polyphosphate, about 10 mM Tris, and has a pH of about 9.

219. The method of claim 201 wherein the anion exchange resin comprises quaternary ammonium groups.

220. The method of claim 219 wherein the anion exchange resin is Q Sepharose.

221. A method of purifying a polypeptide expressed in Escherichia coli, wherein said polypeptide is selected from the group consisting of (i) full length, properly folded human TFPI having the amino acid sequence shown in Figure 4; (ii) full length, properly folded ala-human TFPI having the amino acid sequence shown in Figure 4 and having one further amino acid which is an amino-terminal alanine; and (iii) a full length, properly folded mutein selected from the group consisting of muteins having from 1 to 5 amino acid substitutions of (i) or (ii); the method comprising the steps of:

applying a first preparation comprising said polypeptide to an ion exchange chromatography resin, wherein the first preparation further comprises urea and a charged polymer whose net charge is the same as the net charge of the resin, to bind said polypeptide to the resin;

washing the resin with a wash buffer which is essentially urea-free and comprises the charged polymer; and

washing the resin with an elution buffer which is essentially urea-free and which comprises the charged polymer at a higher concentration than in the wash buffer to elute from the resin a second preparation enriched in said polypeptide compared to said first preparation.

222. The method of claim 221 wherein the polypeptide is full length, properly folded human TFPI having the amino acid sequence shown in Figure 4

223. The method of claim 221 wherein the polypeptide is full length, properly folded ala-human TFPI having the amino acid sequence shown in Figure 4 and having one further amino acid which is an amino-terminal alanine.

224. The method of claim 221 wherein the first preparation comprises urea.

225. The method of claim 224 wherein the first preparation comprises about 3.5 M urea.

226. The method of claim 221 wherein the first preparation has a pH of from about pH 5.5 to about pH 6.0.

227. The method of claim 221 wherein the first preparation comprises about 3.5 M urea, about 50 mM Tris, and has a pH of about 5.9.

228. The method of claim 226 wherein the first preparation further comprises about 1 mg/ml polyphosphate.

229. The method of claim 221 wherein the charged polymer is negatively charged and the resin is a cation exchange resin.

230. The method of claim 229 wherein the charged polymer is polyphosphate.

231. The method of claim 230 wherein the wash buffer comprises about 10 mg/ml polyphosphate.

232. The method of claim 231 wherein the wash buffer has a pH of about 5.

233. The method of claim 231 wherein the wash buffer comprises 10 mM sodium phosphate.

234. The method of claim 230 wherein the elution buffer comprises about 10 mg/ml polyphosphate.

235. The method of claim 234 wherein the elution buffer has a pH of about 7.5.

236. The method of claim 235 wherein the elution buffer comprises 10 mM sodium phosphate.

237. The method of claim 221 wherein the resin is SP Sepharose.

238. The method of claim 221 wherein the charged polymer is positively charged and the resin is an anion exchange resin.

239. A method of purifying a polypeptide expressed in Escherichia coli, wherein said polypeptide is selected from the group consisting of (i) full length, properly folded human TFPI having the amino acid sequence shown in Figure 4; (ii) full length, properly folded ala-human TFPI having the amino acid sequence shown in Figure 4 and having one further amino acid which is an amino-terminal alanine; and (iii) a full length, properly folded mutein selected from the group consisting of muteins having from 1 to 5 amino acid substitutions of (i) or (ii); the method comprising the steps of:

applying a first preparation comprising said polypeptide to an ion exchange chromatography resin, wherein the first preparation further comprises urea and a charged polymer whose net charge is the same as the net charge of the resin, to bind said polypeptide to the resin;

washing the resin with a wash buffer which comprises urea and the charged polymer; and

washing the resin with an elution buffer which comprises urea and which comprises the charged polymer at a higher concentration than in the wash buffer to elute from the resin a second preparation enriched in said polypeptide compared to said first preparation.

240. The method of claim 239 wherein the polypeptide is full length, properly folded human TFPI having the amino acid sequence shown in Figure 4

241. The method of claim 239 wherein the polypeptide is full length, properly folded ala-human TFPI having the amino acid sequence shown in Figure 4 and having one further amino acid which is an amino-terminal alanine.

242. The method of claim 239 wherein the wash buffer comprises about 6 M urea.

243. The method of claim 242 wherein the wash buffer comprises about 6 M urea, about 1 mg/ml polyphosphate, about 10 mM sodium phosphate, and has a pH of about 5.9.

244. The method of claim 239 wherein the elution buffer comprises a gradient of increasing concentration of the charged polymer.

245. The method of claim 244 wherein the gradient is applied over about 25 column volumes.

246. The method of claim 244 wherein the gradient starts at about 1 mg/ml of the charged polymer and ends at about 20 mg/ml of the charged polymer.

247. The method of claim 244 wherein the charged polymer is polyphosphate and the resin is a cation exchange resin.

248. The method of claim 247 wherein the elution buffer comprises a gradient of polyphosphate from about 1 mg/ml to about 20 mg/ml,

249. The method of claim 247 wherein the elution buffer comprises about 6 M urea, a gradient of polyphosphate of from about 1 mg/ml to about 20 mg/ml, 10 mM sodium phosphate, and has a pH of about 5.9.

250. A method to aid in purification of a polypepetide selected from the group consisting of (i) full length, properly folded human TFPI having the amino acid sequence shown in Figure 4; (ii) full length, properly folded ala-human TFPI having the amino acid sequence shown in Figure 4 and having one further amino acid which is an amino-terminal alanine; and (iii) a full length, properly folded mutein selected from the group consisting of muteins having from 1 to 5 amino acid substitutions of (i) or (ii); the method comprising the step of:

adding a second charged polymer to a composition comprising said polypeptide and a first charged polymer, said second charged polymer bearing a net charge which is opposite to the net charge of said first charged polymer, wherein the amount of said second charged polyer added is sufficient to essentially neutralize said first charged polymer.

251. The method of claim 250 wherein the polypeptide is full length, properly folded human TFPI having the amino acid sequence shown in Figure 4

252. The method of claim 250 wherein the polypeptide is full length, properly folded ala-human TFPI having the amino acid sequence shown in Figure 4 and having one further amino acid which is an amino-terminal alanine.

253. The method of claim 250 wherein the first charged polymer is polyphosphate.

254. The method of claim 253 wherein the second charged polymer is polyethyleneimine.

255. A method of purifying a polypeptide expressed in Escherichia coli, wherein said polypeptide is selected from the group consisting of (i) full length, properly folded human TFPI having the amino acid sequence shown in Figure 4; (ii) full length, properly folded ala-human TFPI having the amino acid sequence shown in Figure 4 and having one further amino acid which is an amino-terminal alanine; and (iii) a full length, properly folded mutein selected from

the group consisting of muteins having from 1 to 5 amino acid substitutions of (i) or (ii); the method comprising the steps of:

(a) refolding the polypeptide which is present in a first preparation;

(b) purifying the refolded polypeptide from step (a) by cation exchange chromatography;

(c) further purifying the polypeptide from step (b) by anion exchange chromoatography to form a second preparation enriched in said polypeptide compared to said first preparation.

256. The method of claim 255 wherein the polypeptide is full length, properly folded human TFPI having the amino acid sequence shown in Figure 4

257. The method of claim 255 wherein the polypeptide is full length, properly folded ala-human TFPI having the amino acid sequence shown in Figure 4 and having one further amino acid which is an amino-terminal alanine.

258. A method of purifying a polypeptide expressed in Escherichia coli, wherein said polypeptide is selected from the group consisting of (i) full length, properly folded human TFPI having the amino acid sequence shown in Figure 4; (ii) full length, properly folded ala-human TFPI having the amino acid sequence shown in Figure 4 and having one further amino acid which is an amino-terminal alanine; and (iii) a full length, properly folded mutein selected from the group consisting of muteins having from 1 to 5 amino acid substitutions of (i) or (ii); the method comprising the steps of:

adding polyethylene glycol (PEG) and polyphosphate to a first preparation comprising the polypeptide to form an aqueous two-phase system; and

collecting a PEG-rich phase to form a second preparation enriched in said polypeptide compared to said first preparation.

259. The method of claim 258 wherein the polypeptide is full length, properly folded human TFPI having the amino acid sequence shown in Figure 4

260. The method of claim 258 wherein the polypeptide is full length, properly folded ala-human TFPI having the amino acid sequence shown in Figure 4 and having one further amino acid which is an amino-terminal alanine.

261. The method of claim 258 wherein prior to the step of adding, the polypeptide was obtained by solubilization of inclusion bodies.

262. The method of claim 261 wherein the inclusion bodies were solubilized in a solution comprising about 7 M urea, about 1% (weight/volume) monothioglycerol, about 10 mM CAPS buffer, and having a pH of about 10.

REMARKS

The Amendments

The amendments to the specification correct improper reference to the figures, typographical errors, and update the status of a referenced application. These amendments are supported by the specification or by the drawings and do not introduce any new matter. New claims 71-262 are supported by the specification and the drawings and do not introduce any new matter.

Allowance of all pending claims is respectfully requested.

Respectfully submitted,

Date: November 30, 2001

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MARKED-UP COPY OF SPECIFICATION AND CLAIMS

MARKED-UP REPLACEMENT PARAGRAPHS FOR SPECIFICATION

Page 1, line 1, change the title:

[Method Of Solubilizing, Purifying, And Refolding Protein] Formulation, Solubilization, Purification, and Refolding of Tissue Factor Pathway Inhibitor

page 6, line 16

Figure 5 shows the solubility of TFPI at different pH conditions. About 10 mg/mL TFPI in 2M urea was dialyzed against 20 mM acetate, phosphate, citrate, glycine, L-glutamate [and] or succinate in 150 mM NaCl. The concentration of remaining soluble TFPI after dialysis was measured by UV absorbance after filtering out the precipitates through 0.22 mm filter units.

page 6, line 27

Figure 8 shows effect of pH on the stability of TFPI prepared in 10 mM Na phosphate, 150 mM NaCl and 0.005% (w/v) polysorbate-80. Stability samples containing 150 [mg/mL] µg/mL TFPI were incubated at 40°C for 20 days. Kinetic rate constant for the remaining soluble TFPI was analyzed by following decrease of the main peak on cation exchange chromatograms.

page 7, line 1

Figure 9 shows the percentage of remaining soluble TFPI measured by cation exchange HPLC [(A)] (9A) and remaining active TFPI by prothrombin time assay [(B)] (9B) as a function of phosphate concentration. The formulation contains 150 [mg/mL] µg/mL TFPI prepared in 150 mM NaCl and 0.005% (w/v) polysorbate-80 at pH 7 with varying concentrations of phosphate.

page 7, line 24

Figure 16 shows SDS PAGE analysis of fractions collected during elution of the S-Sepharose HP column used to purify rhTFPI from a polyphosphate-facilitated refold. Fig. 16A

shows Load through Fraction 23, and Fig. 16B shows Fractions 25-39.

Figure 17 shows the absorbance at 280 nm during the loading and elution of the Q-Sepharose HP column used to purify rhTFPI from a S-Sepharose pool prepared from a polyphosphate-facilitated refold.

page 8, line 1

Figure 18 shows SDS PAGE analysis of fractions collected during elution of the Q-Sepharose HP column used to purify rhTFPI from a S-Sepharose pool prepared from a polyphosphate-facilitated refold. Fig. 18A shows Load through Fraction 19, and Fig. 18B shows Fractions 20-24.

Page 8, line 9

Figure 21 shows SDS PAGE analysis of fractions collected during elution of the S-Sepharose HP column used to purify rhTFPI from a polyethyleneimine facilitated refold. Fig. 21A shows Load through Fraction 25, and Fig. 21B shows Fractions 26-Pool after fractions.

page 8, line 15

Figure 23 shows SDS PAGE analysis of fractions collected during elution of the Q-Sepharose HP column used to purify rhTFPI from a S-Sepharose pool prepared from a polyethyleneimine-facilitated refold. Fig. 23A shows Load through Fraction 10, Fig. 23B shows Fractions 11-23, and Fig. 23C shows Fractions 24-30.

page 9, line 23

The invention relates to pharmaceutically acceptable compositions wherein TFPI is present in a concentration of more than 0.2 mg/mL together with solubilizing agents. The solubilizing agents may be acetate ion, sodium chloride, citrate ion, isocitrate ion, glycine, glutamate, succinate ion, histidine, imidazole [and] or sodium dodecyl sulfate (SDS) as well as charged polymers. In some compositions, TFPI may be present in concentrations of more than 1 mg/mL and more than 10 mg/mL. The composition may also have one or more secondary solubilizers. The secondary solubilizer or solubilizers may be polyethylene glycol (PEG),

sucrose, mannitol, or sorbitol. Finally, the composition may also contain sodium phosphate at a concentration greater than 20 mM.

page 14, line 1

Charged polymers can be used to modify the charge, charge density, and reduce or eliminate ionically mediated limitations to conformation that may arise in the unfolded state. The juxtaposition of charged groups that are not normally in proximity may [have] result in dead-end refolding pathways from which the refolding process may never recover.

page 18, line 24

The term "HIC" as used herein refers to hydrophobic interaction chromatography which employs a hydrophobic interaction between the column and the molecule of interest to separate the sulfated polysaccharides and other contaminants from the refolded product.[A.]

page 18, line 28

Negatively charged polymers include sulfated polysaccharides, such as heparins, dextran sulfates, and agaropectins, as well as carboxylic acid polysaccharides such [asalginic] as alginic acids and carboxymethyl celluloses. Polyinorganics such as polyphosphates are also included. Polyamino acids such as polyasparatate, polyglutamate, and polyhistidine can also be used.

page 21, line 3

TFPI can be prepared in yeast expression systems as described in U.S. Serial No. 08/286,530 (now abandoned, but continuation issued as U.S. 6,103,500), which is herein incorporated by reference. Methods have also been disclosed for purification of TFPI from yeast cell culture medium, such as in Petersen *et al*, J.Biol.Chem. 18:13344-13351 (1993). In these cases, recombinant TFPI is secreted from the yeast cell. TFPI recovered in such protocols is also frequently heterogeneous due to N-terminal modification, proteolytic degradation, and variable[.] glycosylation. Therefore, a need exists in the art to produce mature TFPI that is authentic (i.e. having the correct N-terminal amino acid sequence), full-length and homogeneous.

page 22, line 5

TFPI may be prepared by recombinant methods as disclosed in U.S. 5,212,091, the disclosure of which is herein incorporated by reference. Briefly, TFPI is expressed in *Escherichia coli* cells and the inclusion bodies containing TFPI are isolated from the rest of the cellular material. The inclusion bodies are subjected to sulfitolysis, purified using ion exchange chromatography, refolded by disulfide interchange reaction and the refolded, active TFPI purified by cation exchange chromatography. TFPI may also be puroduced in yeast as disclosed in [co-pending U.S.S.N. 08/286,530] U.S. 6,103,500.

TFPI activity may be tested by the prothrombin time assay (PTT assays). Bioactivity of TFPI was measured by the prothrombin clotting time using a model RA4 Coag-A-Mate from Organon Teknika Corporation (Oklahoma City, OK). TFPI samples were first diluted to 9 to 24 ug/mL with a TBSA buffer (50 mM Tris, 100 mM NaCl, 1 mg/mL BSA, pH 7.5) . Then 10 uL of Varify 1 (pooled normal plasma from Organon Teknika Corp.) was mixed with 90 uL of diluted TFPI samples in a sample tray and warmed to [37¡C] 37°C in the instrument. Finally Simplastin Excel (Thromboplastin from Organon Teknika Corp.) was added to start the clotting. The time delay in clotting due to anticoagulant activity of TFPI was measured and converted into TFPI concentration in the measured samples by comparison to a TFPI standard curve.

page 25, line 1

The refolding samples, stored at -20°C, remained in the standard refolding buffer containing 3 M urea, 50 mM Tris, pH 8.8, 1-4 mM redox, 0.5 mg/ml TFPI, and 0.2-0.6 M NaCl depending on condition. Samples refolded with dextran or heparin had 0.2 M salt, and samples without dextran or heparin had 0.6 M NaCl.

page 26, line 21

About 10 mg/mL TFPI in 2M urea was dialyzed against one of the following: 20 mM acetate, 20 mM phosphate, 20 mM citrate, 20 mM glycine, 20 mM L-glutamate or 20 mM succinate in 150 mM NaCl as described above. 6-10 mg/mL TFPI bulk stock was loaded into Spec/Por 7 dialysis tubings (MW cutoff 3,500). Dialysis was carried out either at [4¡C] 4°C or ambient temperature. Three changes of buffer at a protein solution to buffer ratio: 1 to 50-100,

were made during course of dialysis over 12 to 24 hr time period. After dialysis, TFPI solution was filtered by Costar 0.22 micron filter units to separate precipitated TFPI from soluble TFPI. The solubility of TFPI was then measured by UV/Vis absorbance assuming an absorptivity 0.68 $(mg/mL)^{-1}$ cm^{-1} at 278 nm. The solutions were prepared at various pH levels by titration with HCl or NaOH.

page 27, line 1

After completion of dialysis, the precipitates were filtered through 0.22 μm filter units. The concentration of remaining soluble TFPI after dialysis was measured by UV absorbance. [**Figure** 1] Figure 5 shows the results of these experiments. Solubility of TFPI increased greatly in solutions containing 20 mM acetate, 20 mM phosphate, 20 mM L-glutamate and 20 mM succinate at pH levels below 7 and particularly at or below pH 4.5. Solubility of TFPI was also substantially increased in solutions containing 20 mM glycine above pH 10. [**Figure** 2] Figure 6 shows the solubility of TFPI as a function of concentration of citrate ion in the presence of 10 mM Na phosphate at pH 7. TFPI solubility increases with increasing concentration of citrate. Figure [3] 5 shows the solubility of TFPI as a function of concentration of NaCl at pH 7.0. TFPI solubility increases with increasing salt concentration, indicating salt promotes solubility of TFPI.

page 30, lines 37 - 39, column 1

20 mM Na Citrate, 130 mM NaCl, 1% Glycine, 0.25% [Tween-80] Polysorbate-80, 5% PEG-400

20 mM Na Citrate, 130 mM NaCl, 1% Glycine, 0.25% [Tween-80] Polysorbate-80

page 32, line 2

The stability of TFPI stored at various pH conditions was tested. TFPI was prepared by dialysis as above in 10 mM Na phosphate, 150 mM NaCl and 0.005% (w/v) polysorbate-80. Stability samples containing 150 mg/mL TFPI were incubated at [40¡C] 40°C for 20 days. Kinetic rate constant for the remaining soluble TFPI was analyzed by following decrease of the main peak on cation exchange chromatograms. As can be seen in [**Figure 5**] Figure 8, the decay

rate constant increases at pH above 6.0, indicates more aggregation at higher pH conditions.

page 32, line 9

TFPI was also formulated at a concentration of 150 mg/mL in 150 mM NaCl and 0.005% (w/v) polysorbate-80 at pH 7 with varying concentrations of phosphate. [**Figure 5A**] Figure 9A shows the percentage of remaining soluble TFPI measured by the cation exchange HPLC. Increasing concentrations of phosphate ion in solution resulted in higher levels of soluble TFPI remaining after incubation at 40°C. Higher levels of phosphate ion also resulted in higher levels of active TFPI as assayed by the prothrombin time assay. These results are shown in [**Figure 5B**] Figure 9B.

Stability of TFPI at a concentration of 0.5 mg/mL and formulated in 10 mM Na citrate, pH 6 and 150 mM NaCl was also tested at 40°C over a [40] 20 day period. As seen in [**Figure 6**] Figure 10, cation-exchange HPLC (triangle) shows the presence of soluble TFPI at levels greater than 60% initial, even after the [40] 20 day incubation. In like manner, the prothrombin time assay (circle) shows the presence of active TFPI at levels greater than 60% initial, even after the [40] 20 day incubation.

page 32, line 22

Figure [7] 11 shows loss of soluble TFPI at 40°C measured by both cation-exchange HPLC (open symbol) and prothrombin time assay (closed symbol) for 0.5 mg/mL TFPI formulated in 10 mM Na phosphate, pH 6 and either 150 mM NaCl (triangle) or 500 mM NaCl (circle).

page 32, line 26

Figure [8] 12 shows loss of soluble TFPI at 40°C measured by both cation-exchange HPLC (open symbol) and prothrombin time assay (closed symbol) for 0.5 mg/mL TFPI formulated in 10 mM Na acetate and pH 5.5 containing 150 mM NaCl (triangle) or 8% (w/v) sucrose (square) or 4.5% (w/v) mannitol (circle).

page 33, line 1

Figure [9] 13 shows two non-reducing SDS gels for TFPI formulation samples in 10 mM $NaPO_4$, 150 mM NaCl, and 0.005% polysorbate-80 at pH 4 to pH 9 stored at 40°C for 0 days (lower) and 20 days (upper). No loss on TFPI is seen at 0 days. However, at 20 days cleavage fragments of TFPI may be seen at the lower pH range (i.e. pH 4 and pH 5). Without being bound to a particular theory, it is believed that these fragments may result from an acid catalyzed reaction.

page 37, line 5

The acidified, filtered refold was loaded onto the equilibrated SP Sepharose HP column at a flow rate of approximately 80.0 ml/min. Flow rate was adjusted to accommodate overnight loading of the acidified filtered refold mixture. The column was equilibrated in 6 M urea, 20 mM sodium phosphate buffer pH 5.9 prior to loading. After loading, the column is washed with 2 [CF] CV of 6 M urea, 0.3 M NaCl, 20 mM sodium phosphate buffer, pH 5.9 prior to the gradient elution step. The column flow rate was increased to 190-200 ml/min for the wash step and all subsequent steps (linear velocity =~ 47 cm/hr). The product was eluted from the column using a linear salt gradient from 0.3 to 0.5 M NaCl in 6 M urea, 20 mM sodium phosphate buffer, pH 5.9. The gradient was formed by delivering 6 M urea, 0.5 M NaCl, 20 mM sodium phosphate buffer into 6 M urea, 0.3 M NaCl, 20 mM sodium phosphate buffer. Limit buffer was pumped with a Masterflex pump (model 7553-20) with a Masterflex head (model 7015.21) at a flow rate of approximately 100 ml/min. with vigorous mixing using a Paratrol A mixer from Parametrics (model 250210). The total volume of the gradient was 71.0 liters or 13.0 CV. The pH of the gradient buffers was 5.92 (+/- .02). Fractions are evaluated qualitatively using SDS PAGE and pooled based on the content of the correctly folded SC-59735 relative to other misfolds and impurities. After pooling the process stream is referred to as the S pool.

page 38, line 3

An Amicon column (7.0 cm diameter) was packed with approximately 700 ml of Q-Sepharose high performance medium (Pharmacia Q-Sepharose HP). The column was packed with 20% ethanol at 20 psi. The bed height after packing was approximately 18 cm. The column was equilibrated with 5 [CF] CV of 6 M urea, 0.02 M Tris/HCl buffer, pH 8. The target

for protein loading is 8-10 mg protein/ml Q Sepharose resin. The Q load was applied to the column at a flow rate 30-35 ml/min (50 cm/hr). After loading, the column was washed with approximately 5 CV of 6 M urea, 20 mM Tris/HCl buffer, pH 8.0, or until the absorbance at 280 nm returned to baseline. The product was eluted using a sodium chloride gradient from 0-0.15 M NaCl in 6 M urea, 20 mM Tris/HCl buffer, pH 8.0 over 25 column volumes. The first seven column volumes were collected as a single fraction, followed by 30 fractions of 0.25 column volume each.

page 39, line 24

After approximately 96 h, the refolding process was terminated by adjusting the pH of the refold to pH 5.9 using glacial acetic acid. Stirring was continued for 90 minutes and the pH checked. More acid was added, if necessary to adjust the pH to 5.9 [/-] +/- 0.1.

page 41, line 6

The pH of the S-pool was next adjusted to pH 8.0 with 2.5 N NaOH. The S Pool was concentrated 2-3 fold to approximately 2 L using an Amicon DC-10L ultrafiltration unit containing an Amicon YM10 spiral cartridge (10,000 [N.W.] M.W. cut-off membrane). After concentration, the concentrated S Pool was diafiltered against 7 volumes of 6 M urea, 20 mM Tris-HCl buffer, pH 8.0. The diafiltration was considered complete when the conductivity of the retentate was below 2 mS. The diafiltered concentrate was drained from the ultrafiltration unit and the unit was washed with approximately 1 L of diafiltration buffer. The [was] wash is combined with the concentrate to form the Q-load.

page 41, line 15

An Amicon column ([7.0o] 7.0 cm diameter) was packed with approximately 700 ml of Q-Sepharose high performance medium (Pharmacia Q-Sepharose HP). The column was packed in 20% ethanol at 20 psi. The bed height after packing was approximately 18 [c.m] cm. The column was equilibrated with 5 CV of 6 M urea, 0.02 M Tris/HCl buffer, pH 8. The target for protein loading is 8-10 mg protein/ml Q Sepharose resin. The Q load was applied to the column at a flow rate 30-35 ml/min (50 cm/hr). After loading, the column was washed with

approximately 5 CV of 6 M urea, 20 mM Tris/HCl buffer, pH 8.0, or until the absorbance at 280 nm returned to baseline. The product was eluted using a sodium chloride gradient from 0-0.15 M NaCl in 6 M urea, 20 mM Tris/HCl buffer, pH 8.0 over 25 column volumes. The first seven column volumes were collected as a single fraction, followed by 30 fractions of 0.25 column volume each.

page 42, line 1

The Q-Sepharose fractions to be pooled were thawed by incubation at 2-8°C, pooled, and the pH of the pool was adjusted to 7.2 using 2 [MHCl] M HCl. The pool was then concentrated approximately 5 fold in an Amicon DC-1 ultrafiltration system containing a S1Y1 Amicon YM-10 cartridge (10,000 MWCO spiral cartridge membrane). The concentrated Q Pool was then diafiltered against seven column volumes of 2 M urea, 0.15 M NaCl, 20 mM sodium phosphate buffer, pH 7.2. Following ultrafiltration, the solution was drained from the ultrafiltration system. Approximately 100 ml of 2 M urea, 0.15 M NaCl, 20 mM sodium phosphate buffer, pH 7.2 was circulated through the ultrafiltration system for approximately 5 min. The rinse solution was combined with the original concentrate and filtered through a 0.45 micron vacuum filter unit (Nalgene).

page 42, line 16

About 2 g of rhTFPI (43 ml inclusion body slurry containing 46 mg/ml rhTFPI) was dissolved with mixing in 4 L of 50 mM Tris buffer, pH 10.5 containing 4 g/l polyphosphate (Glass H, FMC Corporation) at 2-8°C. Sufficient cysteine and cystine was added to make the solutions 0.1 mM and 0.05 mM respectively. The pH was maintained at pH 10.5 with 1 N NaOH. The refold solution was incubated at 2-8°C with gentle mixing for 72-96 h.